**Graphene Oxide, But Not Fullerenes, Targets Immunoproteasomes And Suppresses Antigen Presentation By Dendritic Cells**

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## Supporting information

### Experimental Section

*Nanoparticles*. Sublimed $C_{60}$ (99.9% purity, HPLC) and $C_{60}$-pyrrolidine tris-acid ($C_{60}$-TRIS) (95.0% purity, HPLC) were purchased from Sigma (Sigma-Aldrich, Saint Louis, MO). Graphene oxide was synthesized as described elsewhere.[1]

*Particle characterization.* The CNP used in the present study were characterized by different means. Diffuse reflectance infrared Fourier Transform spectroscopy (DRIFTS) was performed on $C_{60}$ fullerenes and $C_{60}$-TRIS that were homogeneously mixed with KBr. A spectrum was obtained over the range of 400 to 4000 $cm^{-1}$ with a resolution of 4 $cm^{-1}$ using an IR-Prestige spectrophotometer (Shimadzu Scientific) outfitted with an EasiDiff accessory (Pike Technologies), with KBr as the background taking 32 scans per sample. Major peaks for $C_{60}$ fullerenes were observed at 527, 575, 1182, and 1429 $cm^{-1}$ (Figure S1), which corresponds well with the values determined by Krätschmer *et* al.[2,3] The weaker vibration at 1537 $cm^{-1}$ has been observed in the infrared spectrum of $C_{60}$.[4] Finally, the feature at 2326 $cm^{-1}$ was attributed to the asymmetric stretch of $CO_2$.[5] For $C_{60}$-TRIS, significant peaks occurred at 3462 $cm^{-1}$ and 1734 $cm^{-1}$ (Figure S1), and were attributed to the O-H and C=O stretches of the carboxylic acids groups[6]. Furthermore, the peaks at 1217 $cm^{-1}$ corresponded to the C-O stretch, and the peaks at 527 $cm^{-1}$ and 1192 $cm^{-1}$ were comparable to features in the spectrum for $C_{60}$.[6] GO particles used in the current study were characterized previously.[1]

The zeta potential was determined on a Malvern Zetasizer Nano instrument (Malvern Instruments, Westborough, MA) by Particle Technology Labs, Ltd. (Dowers Grove, IL). The three samples ($C_{60}$, $C_{60}$-TRIS, and GO) were originally suspended in pH 7.0 PBS at a concentration of 1 mg/ml. For ζ potential measurements, the samples were diluted to 0.1 mg/ml with PBS at pH 7.0, sonicated utilizing a probe tip sonicator (three one minute cycles), and filtered employing a 1 μm filter. The ζ potential was -13.6, -26.1, and -32.4 mV for $C_{60}$ fullerenes, $C_{60}$-TRIS fullerenes, and GO, respectively. The zeta potential values for $C_{60}$ fullerenes and GO were consistent with literature reports.[1,6]

Particle size measurements were conducted employing a Malvern Mastersizer 2000 (Malvern Instruments, Westborough, MA) on three samples ($C_{60}$ fullerenes, $C_{60}$-TRIS fullerenes, and GO) dispersed in pH 7.0 PBS at a concentration of 1 mg/ml by Particle Technology Labs, Ltd. Analysis was conducted according to standard operating procedure for this instrumentation.

Scanning electron microscopy was implemented utilizing a SEM-JSM6510LV (JEOL Ltd., Tokyo, Japan). Suspensions $C_{60}$ fullerenes, $C_{60}$-TRIS, or GO were spin-coated on a section of silicon wafer. The samples were analyzed employing a working distance of 10 to 15 mm and an accelerating voltage that ranged between 15 to 20 kV. To perform the transmission electron microscopy (TEM), one drop of the sample (0.1 mg/ml) was placed on a lacey carbon grid (Pacific-Grid Tech) and allowed to dry in ambient conditions for 2 hours prior to TEM imaging (FEI Morgagni, 80 keV). The size distributions obtained by TEM were 45.2±25.3 nm for $C_{60}$ fullerenes and 45.6±18.8 nm for $C_{60}$-TRIS particles (Figure S2). GO sheet heights were ~ 0.61 nm, while size averaged at 0.5±0.35 μm.[6]

Energy-dispersive X-ray Spectroscopy (EDS) was employed to characterize the elemental composition of the $C_{60}$ fullerene, $C_{60}$-TRIS fullerene, and GO samples.[7] The elemental spectrum for $C_{60}$ contained 74.25 at.% C, 19.65 at.% O, 2.22 at.% Na, 0.99 at.% Si, 0.62 at.% S, 2.27 at.% Cl. For $C_{60}$-TRIS, the elemental spectrum consisted of 77.45 at.% C, 18.47 at.% O, 2.02 at.% Na, and 2.06 at.% Cl; the Na and

Cl contribution was attributed to PBS. The elemental analysis of GO revealed 61.05 at.% C, 26.74 at.% O, and 12.21 at.% Si from the silicon substrate.

*Cell cultures and generation of DCs*. B3Z hybridoma T cells were cultured in complete RPMI 1640 medium supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES (10 mM), heat-inactivated FBS (10%), nonessential amino acids (0.1mM), and sodium pyruvate (1mM, Invitrogen Life Technologies) (complete medium). B3Z T cells were maintained and used as described by Pulaski *et al.*[8]

Bone marrow-derived DCs were generated as previously described.[9] Briefly, bone marrow cells were collected from tibias and femurs of C57BL/6 mice, depleted of red blood cells with lysing buffer for 3 min. The single-cell suspensions were then incubated with anti-mouse B220, CD4 and CD8 antibodies for 1 h at 4°C followed by incubation with guinea pig complement for 30 min at 37°C to deplete B and T lymphocytes. Cells were then cultured overnight (37°C, 5% $CO_2$) in six-well plates at the concentration of 1 x $10^6$ cells/ml in complete RPMI-1640 medium. The nonadherent cells were collected and seeded at a concentration of 2 x $10^5$ cells/ml in six-well plates in complete medium in the presence of recombinant mouse GM-CSF (1000 U/ml) and IL-4 (1000 U/ml) (PeproTech, Rocky Hill, NJ).

*Antigen-specific priming of T cell responses*. To investigate if exposure of DCs to CNP affects the antigen-specific priming of T cells, cultured bone marrow-derived DCs were exposed to GO, $C_{60}$ or $C_{60}$-TRIS or vehicle for 18 h on day 5 of DC culture. Further, DCs were loaded with chicken egg ovalbumin (OVA, 1.0 mg/ml, Hyglos GmbH, Regensburg, Germany) for 16 h. Alternatively, DCs were exposed to CNP (1.0, 6.25, or 25 μg/ml) after OVA loading. Following incubation with CNP and/or OVA loading, DCs were mixed with B3Z hybridoma T cells (DC:B3Z - 1:2) recognizing SIINFEKL peptide in the context of MHC class I molecules. IL-2 content in supernatants, reflecting activation of antigen-specific

T cells, was measured utilizing a commercially available mouse IL-2 ELISA (R&D Systems, Inc., Minneapolis, MN) following 48h of co-culture. To investigate whether CNP exposure modifies the peptide antigen presentation without antigen processing, we utilized the same protocol for CNP exposure; however, DCs were loaded with $OVA_{257-264}$ SIINFEKL peptide (AnaSpec Inc., Fremont, CA) (0.01 μM, 4 h). Negative controls, including non-OVA (or peptide)-loaded DCs exposed to CNP or vehicle, were used for background readings.

*Measurements of OVA and Lucifer Yellow engulfment by DCs.* To assess if CNP exposure interferes with the ability of DCs to capture model protein antigen (OVA) or inert dye (Lucifer Yellow), i.e., pinocytosis, cultured DCs were pre-exposed to CNP (1.0, 6.25, or 25 μg/ml) as described above and loaded with FITC-labeled OVA (1.0 mg/ml, Invitrogen, Carlsbad, CA) or Lucifer Yellow dye (1.0 mg/ml, Invitrogen, Carlsbad, CA) for 30 min at 37°C or 4°C, respectively. Following OVA/dye loading, DCs were washed 4 times to remove residual free OVA/dye and immediately analyzed by flow cytometry. Mean fluorescence intensity at 540 nm excitation wavelength was recorded, and the readings from the samples incubated at 4°C were subtracted from the ones incubated at 37°C.

*Expression of antigen-processing machinery (APM) components in DCs*. To assess if CNP exposure has effects on APM components in DCs, cultured DCs were pre-exposed to CNP (6.25 μg/ml) as described above. Cells were washed in PBS containing 1% (w/v) BSA and placed in tubes ($2 \times 10^5$ cells/tube). Cells were fixed with 2% (w/v) paraformaldehyde for 20 min at room temperature, washed extensively in PBS-BSA, resuspended in 10 ml of PBS-BSA buffer, transferred into Pyrex flasks (Corning Glass, Corning, NY), and subjected to the microwave treatment for 60 s at low power (<700 W) for antigen retrieval. Cells were then chilled on ice for 10 min, washed, and permeabilized, using 0.1% (w/v) saponin in the PBS-BSA buffer and incubated with the primary antibodies recognizing the following APM components: LMP1, LMP7, LMP10, ERp57, calreticulin, TAP-1 and calnexin (at 10–25 μg/ml

saponin-containing buffer) for 30 min at room temperature. Antibodies to APM components were characterized earlier.[10] After being washed with three changes of the saponin buffer, cells were incubated with optimally pretitred goat FITC-conjugated anti-mouse IgG antibodies for 30 min at room temperature. Cells were then washed twice in saponin buffer, fixed with 1% (w/v) paraformaldehyde, and analyzed by flow cytometry. Data are presented as median fluorescence intensity.

*Detection of DC phenotype.* To evaluate if CNP are capable of inducing phenotypical maturation of DCs, cultured DCs were exposed to CNP (6.25 μg/ml) as described earlier and stained with antibodies (Biolegend Inc., San Diego, CA) directly conjugated with FITC or PE. Expression of CD80, CD86, CD40, MHCII (IAb) and MHC I ($H^2Kb$) was measured on CD11c+ cells using the BD FACS Calibur instrument. Data were expressed as the mean fluorescence intensity (MFI).

*Molecular Modeling Studies.* The structures of C60 fullerene and GO were docked to the LMP7 using Autodock Vina.[11] The structure of the LMP7 protein was extracted from the Modbase database, which is a homology model generated based on the protein as a template and the structures of C60 fullerene and GO were generated using the Nanotube Modeller Software (http://www.jcrystal.com/products/wincnt/ index.htm). AutoDockTools (ATD) package (http://autodock.scripps.edu/resources/adt) was further used for formatting and converting the pdb structure files to pdbqt format. The docking was performed using the center of the protein LMP7 as the grid center with x, y, and z centers as 5.6, 80.9, and 41.7, respectively and a grid box of size 60Å x 60Å x 60Å. The resulting binding poses were clustered together and the conformation with the lowest predicted binding energy was considered for further analysis in each case.

*Statistical analysis.* The data were compared by one-way ANOVA and Student's unpaired *t*-test with Welch's correction for unequal variances. All results are presented as the means ± SEM. P values of < 0.05 were considered to be statistically significant. All experiments were repeated at least twice.

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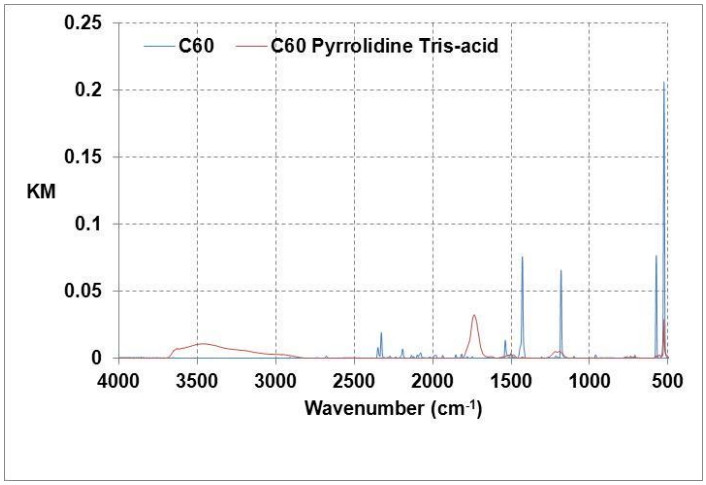


**Figure S1.** Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) performed on $C_{60}$ fullerenes and $C_{60}$ pyrrolidine tris-acid fullerenes. The unit for the ordinate axis is Kubelka-Munk (KM).

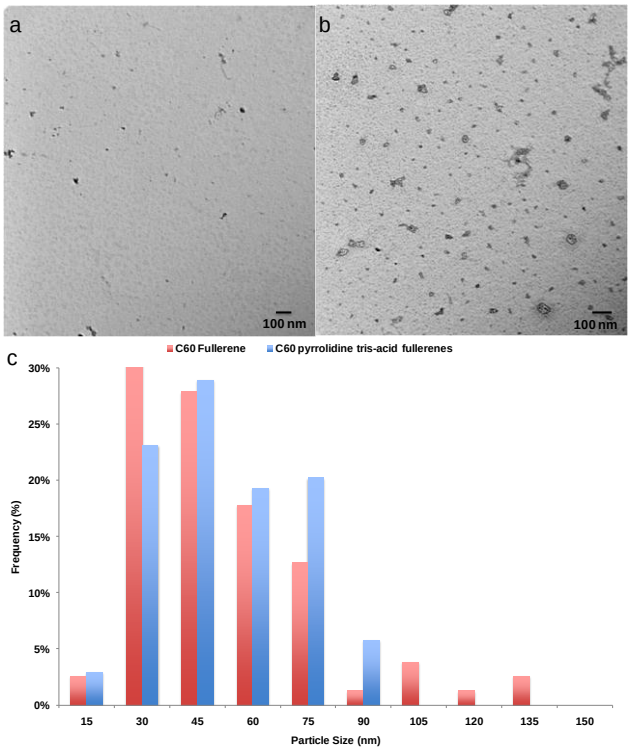


**Figure S2.** TEM micrographs for $C_{60}$ (A), $C_{60}$-TRIS (B). Particle size distribution for $C_{60}$ and $C_{60}$-TRIS (C).